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Cartilage-Derived Morphogenetic Proteins and Osteogenic Protein-1 Differentially Regulate Osteogenesis

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ABSTRACT

Cartilage-derived morphogenetic proteins-1 and -2 (CDMP-1 and CDMP-2) are members of the bone morphogenetic protein (BMP) family, which play important roles in embryonic skeletal development. We studied the biological activities of recombinant CDMP-1 and CDMP-2 in chondrogenic and osteogenic differentiation and investigated their binding properties to type I and type II serine/threonine kinase receptors. In vivo, CDMP-1 and CDMP-2 were capable of inducing dose-dependently de novo cartilage and bone formation in an ectopic implantation assay. In vitro studies using primary chondrocyte cultures showed that both CDMP-1 and CDMP-2 stimulated equally de novo synthesis of proteoglycan aggrecan in a concentration-dependent manner. This activity was equipotent when compared with osteogenic protein-1 (OP-1). In contrast, CDMPs were less stimulatory than OP-1 in osteogenic differentiation as evaluated by alkaline phosphatase activity and expression levels of bone markers in ATDC5, ROB-C26, and MC3T3-E1 cells. CDMP-2 was the least osteogenic in these assays. Receptor binding studies of CDMP-1 and CDMP-2 revealed that both have affinity for the BMP receptor type IB (BMPR-IB) and BMPR-II, and weakly for BMPR-IA. Moreover, using a promoter/reporter construct, transcriptional activation signal was transduced by BMPR-IB in the presence of BMPR-II upon CDMP-1 and CDMP-2 binding. Our data show that distinct members of the BMP family differentially regulate the progression in the osteogenic lineage, and this may be due to their selective affinity for specific receptor complexes. (J Bone Miner Res 1998;13:383–392)

INTRODUCTION

CARTILAGE-DERIVED morphogenetic proteins-1 and -2 (CDMP-1 and CDMP-2) are two members of the transforming growth factor-β (TGF-β) superfamily. During development they are expressed predominantly in and around skeletal elements and in the joint interzones.[1,2] CDMP-1 and CDMP-2 are 82% identical in their biologically active carboxy-terminal domains. They are most closely related to the bone morphogenetic protein (BMP) subgroups BMP-5/BMP-6/osteogenic protein-1 (OP-1) and BMP-2/BMP-4.[1]

The physiological role of CDMP-1, the human homolog of mouse growth differentiation factor-5 (Gdf-5), has been established by its linkage to mouse and human skeletal disorders, brachypodism, and Hunter-Thompson chondrodysplasia, respectively.[1–3] Both phenotypes are characterized by skeletal abnormalities restricted to the limbs and synovial joints and are associated with null mutations in the *cdmp1/gdf5* gene. These genetic studies provide direct evidence for the involvement of CDMP-1 in the patterning and tissue specification of the appendicular skeletal structures. The primary role of CDMP-2, the human homolog of mouse Gdf-6, is so far unknown. Besides its involvement in skeletal morphogenesis, its high levels of expression in

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postnatal cartilaginous tissues suggest a possible role in the promotion and maintenance of the cartilaginous phenotype.[1,4]

TGF-β superfamily members elicit their biological response through binding to a heteromeric complex of two types of serine/threonine kinase receptors (i.e., type I and type II). Various type I and type II receptors for BMPs have been identified.[5] Receptor binding studies have revealed that BMPs have an affinity for several distinct receptor complexes.[6] Interestingly, as shown for OP-1, BMPs appear to utilize different heteromeric receptor complexes to elicit their diverse biological functions.[7]

The expression pattern of the CDMPs is restricted to skeletal structures during embryonic development and therefore suggests a more defined role in the regulation of chondrogenic and osteogenic differentiation. Therefore, we have investigated skeletal lineage progression using recombinant CDMP-1 and CDMP-2 and compared their activities with OP-1.[8] Our data show that both CDMPs and OP-1 promote chondrogenesis; however, CDMPs are significantly less osteogenic than OP-1. Furthermore, receptor binding studies indicate that the distinct biological profile of the CDMPs when compared with OP-1 may be explained by their selective affinity for specific heteromeric receptor complexes.

MATERIALS AND METHODS

Expression of CDMP-1 and CDMP-2 in Escherichia coli

A cDNA encoding the mature *cdmp-1* was tailored for insertion into an *E. coli* expression vector by site-directed mutagenesis using the Kunkel method.[9] Following the pro-domain and in close proximity to the RXXR processing site, a leucine residue was converted to a methionine translational initiation codon with a corresponding *Nco*I restriction site, while a 3× *Xho*I site was similarly introduced immediately after the translational stop codon. The *Nco*I-*Xho*I fragment containing the open reading frame for mature *cdmp-1* was ligated with a tetracycline-resistant pBR322-derived expression vector. Fermentation was done in shaker flasks using 2YT medium with the addition of indol acrylic acid at the appropriate time for the induction of the tryptophan promoter and led to accumulation of large inclusion bodies. A cDNA for *cdmp-2* was similarly tailored for expression. Since the yield of expression was quite low, we used part of the N-terminal region of the highly expressed *cdmp-1* and spliced it with the 7-cysteine domain of *cdmp-2* at the first cysteine, where both genes share a *Pst*I site. Induced cell cultures (250 ml) were centrifuged (11,000*g* for 10 minutes at 4°C), followed by resuspension of the cell pellets in 50 ml of 25 mM Tris, 10 mM EDTA, pH 8.0 (1× TE) plus 100 μg/ml lysozyme. The cell suspensions were incubated overnight at 37°C, then chilled on ice and disrupted by sonication. Inclusion bodies were isolated by centrifugation (11,000*g* for 20 minutes at 4°C) and resuspension in 1× TE. The final washed inclusion body pellets were resuspended in 40 ml of 1× TE, 15% glycerol, and stored at –20°C.

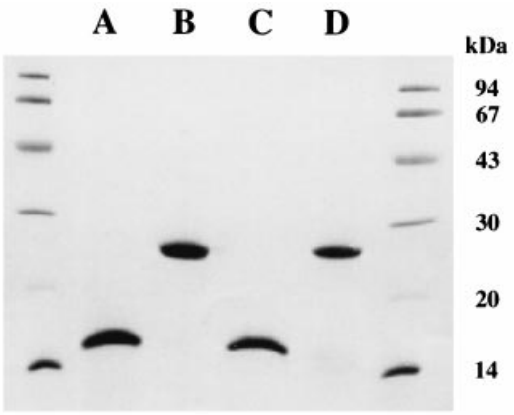

FIG. 1. SDS-PAGE analysis of recombinant CDMP-1 and CDMP-2. Lane A, 5 μg CDMP-1 after reduction and alkylation; lane B, 5 μg CDMP-1 nonreduced; lane C, 5 μg CDMP-2 after reduction and alkylation; lane D, 5 μg CDMP-2 nonreduced.

Protein folding and protein purification

Reduced and denatured inclusion body protein solutions were prepared by dissolving aliquots of pelleted inclusion bodies in 100 mM Tris, 10 mM EDTA, 6 M guanidine HCl, 10 mM dithiothreitol, pH 8.0 (final protein concentration 4-6 mg/ml). The inclusion body protein solutions were incubated at 37° C for 30 minutes then diluted 40-fold with refolding buffer (100 mM Tris, 10 mM EDTA, 1 M NaCl, 2% 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 5 mM reduced glutathione, 2.5 mM oxidized glutathione, pH 8.7). The refolding reactions were incubated for 72 h at 4°C. The folding reactions were dialyzed extensively against 10 mM HCl, then clarified by centrifugation (11,000*g* for 20 minutes at 4°C). The solutions were concentrated using a stirred cell concentrator and YM10 MWCO membranes (Amicon, Beverly, MA, U.S.A.). The concentrated proteins were then lyophilized. The lyophilized proteins were resuspended in 0.8 ml 0.1% trifluoracetic acid (TFA), 70% acetonitrile, then diluted to 2 ml with 0.1% TFA (final acetonitrile concentration 30%). The protein solutions were fractionated by semipreparative C4 reverse phase-high pressure liquid chromatography (HPLC) using a linear acetonitrile gradient (30–100% in 0.1% TFA). Aliquots of each fraction were analyzed on SDS-PAGE (15%) gels in nonreduced conditions and after reduction and alkylation. Peak dimer fractions were pooled and ultraviolet absorbance spectra obtained. Concentrations were estimated from absorbance at 280 nm. Protein pools were stored at –20°C. The average yield of properly folded protein per liter of bacterial fermentation were ~50–60 mg for CDMP-1 and 40–50 mg for CDMP-2.

In vivo subcutaneous implantation assay

To evaluate the potential of CDMP-1 and CDMP-2 to induce cartilage and bone at nonskeletal sites in vivo, in-

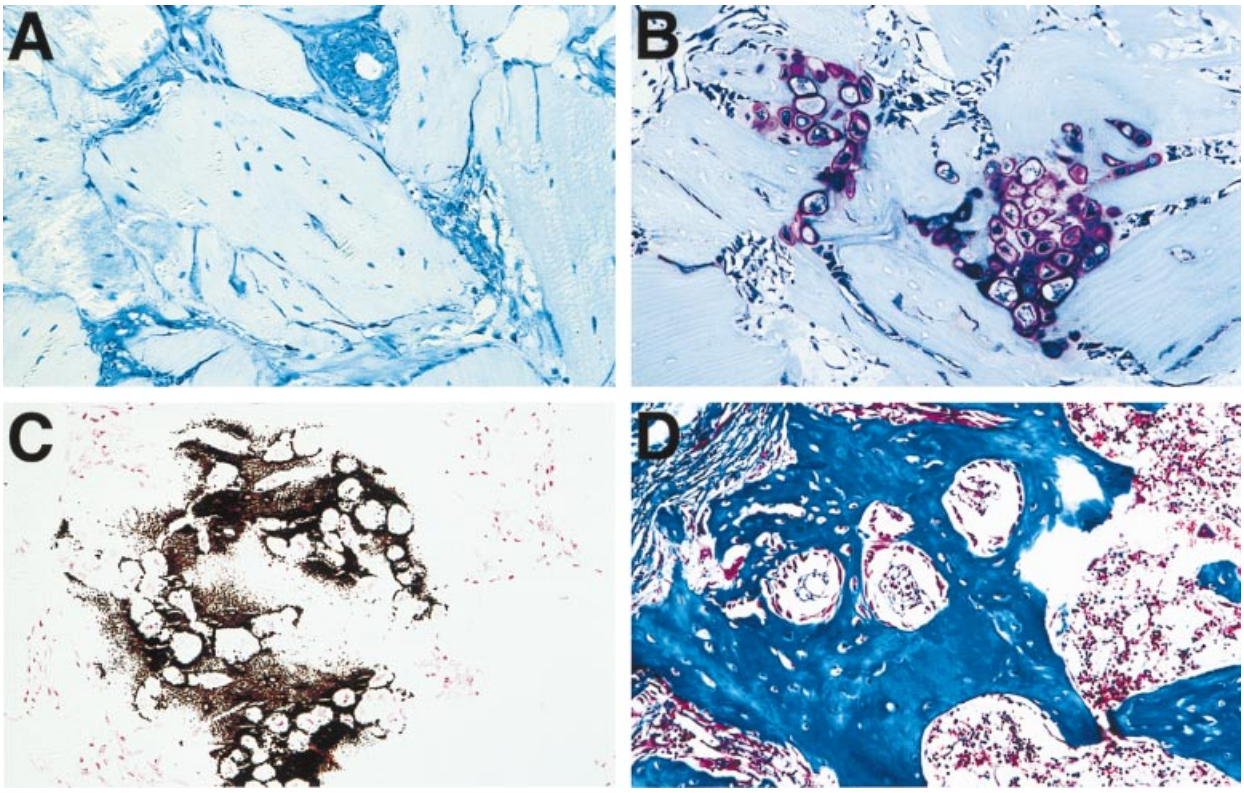

FIG. 2. In vivo ectopic induction of cartilage and bone by CDMPs. A) Negative control consisting of guanidine-extracted demineralized rat bone matrix (residue used as carrier; day 10; toluidine blue staining). (B) One microgram of CDMP-1-treated tissue showing newly formed cartilage islands between the rat carrier matrix particles (day 10; toluidine blue staining). (C) One microgram of CDMP-1-treated tissue showing Von Kossa staining of implants after 10 days. (D) One microgram of CDMP-1-treated tissue showing Masson's trichrome staining of newly formed woven bone and bone marrow in implants after 21 days. (A, B, and C) Magnification ×400; (D) ×200.

creasing doses of recombinant growth factors were reconstituted with 25 mg of rat collagen carrier, lyophilized, and implanted subcutaneously in the thoracic region of 28- to 35-day-old male Long-Evans rats as described.[10] Implants without the addition of CDMPs served as negative controls. The animals were sacrificed 10 and 21 days after implantation, and their implants were fixed, plastic embedded, and sectioned. Sections were subsequently stained with toluidine blue, von Kossa, or Masson's trichrome. Cartilage and bone-forming activity were further quantitated by determining the specific activity of alkaline phosphatase (ALP).[10]

Cell culture

The mouse embryonic teratocarcinoma cell line ATDC5, the mouse calvarial osteoblastic clonal cell line MC3T3-E1, the mouse myoblast cell line C2C12, and R mutant Mv1Lu cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 containing 5% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies, Gaithersburg, MD, U.S.A.). The rat osteoprogenitor-like cell line ROB-C26 was grown in alpha-minimal essential medium (Life Technologies) containing 10% FBS and antibiotics. All experiments were performed under serum-free conditions using a chemically defined basal medium (BM). The serum-free BM consisted of Ham's F-12/DMEM (1/1) with ITS + culture supplement (Collaborative Biomedical Products, Bedford, MA), alpha-ketoglutarate (1×10^{-4} M), ceruloplasmin (0.25 U/ml), cholesterol (5 μg/ml), phosphatidylethanolamine (2 μg/ml), alpha-tocopherol acid succinate (9×10^{-7} M), reduced glutathione (10 μg/ml), taurine (1.25 μg/ml), triiodothyronine (1.6×10^{-9} M), hydrocortisone (1×10^{-9} M), and parathyroid hormone (5×10^{-10} M), β-glycerophosphate (10 mM final concentration), and L-ascorbic acid 2-sulfate (50 μg/ml) (Sigma Chemical Co., St. Louis, MO, U.S.A.).

For proteoglycan biosynthesis and ALP activity assays, cells were plated at a density of 4×10^4 cells in BM in 24-multiwell plates (Costar, Cambridge, MA, U.S.A.). Growth factors were added the next day, and the culture media were replaced every other day. Cultures were maintained at 37°C in humidified air and 5% CO_2. To determine DNA synthesis, 1×10^6 cells were plated out in BM in 100 mm tissue culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) and cultured similarly.

Human fetal limbs from 52- to 79-day-old fetuses were kindly provided by the Central Laboratory for Human Embryology, University of Washington, Seattle, WA, U.S.A..

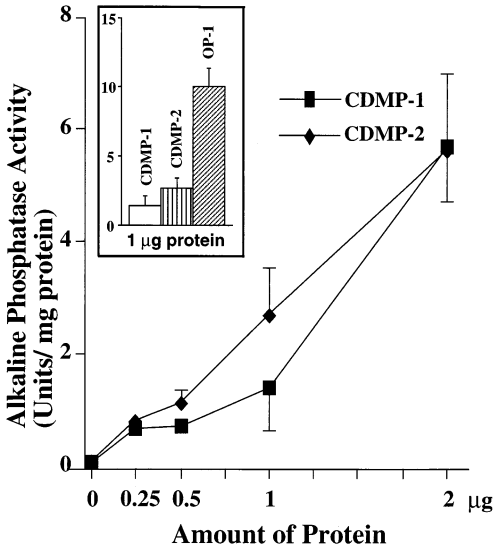

FIG. 3. ALP activity in CDMP-1 and CDMP-2 containing in vivo implants. ALP activity was measured to determine the extent of chondro/osteogenesis induced by increasing concentrations of purified refolded bacterially expressed CDMP-1 and CDMP-2. Values represent the means and standard deviations of the means of four to eight observations. Comparison of ALP activity between equal amounts (1 μg) of CDMP-1, CDMP-2, and OP-1 is depicted in the inset. Experiments were repeated three times.

This was approved by the Office of Human Subjects Research of the National Institutes of Health. The cartilaginous cores were carefully dissected from the surrounding fetal tissue, and the chondrocytes were released by a 6-h digestion in 0.2% collagenase B (Boehringer Mannheim, Indianapolis, IN, U.S.A.) in BM at 37°C. Postnatal bovine articular chondrocytes were prepared as described.[11] For the evaluation of newly synthesized proteoglycans and for DNA-content measurement, chondrocytes were plated out in BM in the same density as described above for the cell lines.

DNA determination

DNA content from chondrocytes and ATDC5 cells was determined after 1, 2, and 10 days of culture using bisbenzimide (Hoechst 33258, Sigma).[12]

ALP activity

ALP activity was determined in sonicated cell homogenates after 4, 6, and/or 10 days of treatment.[13] Briefly, after extensive washing with phosphate buffered saline (PBS), cell layers were sonicated in 500 μl of PBS containing Triton X-100 (0.05% final concentration). Aliquots of 50–100 μl were assayed for enzyme activity in assay buffer (0.1 M sodium barbital buffer, pH 9.3) and p-nitrophenyl phosphate (Sigma) as substrate. Absorbance was measured at 400 nm. Activity was normalized to protein content measured by the Bradford protein assay using bovine serum

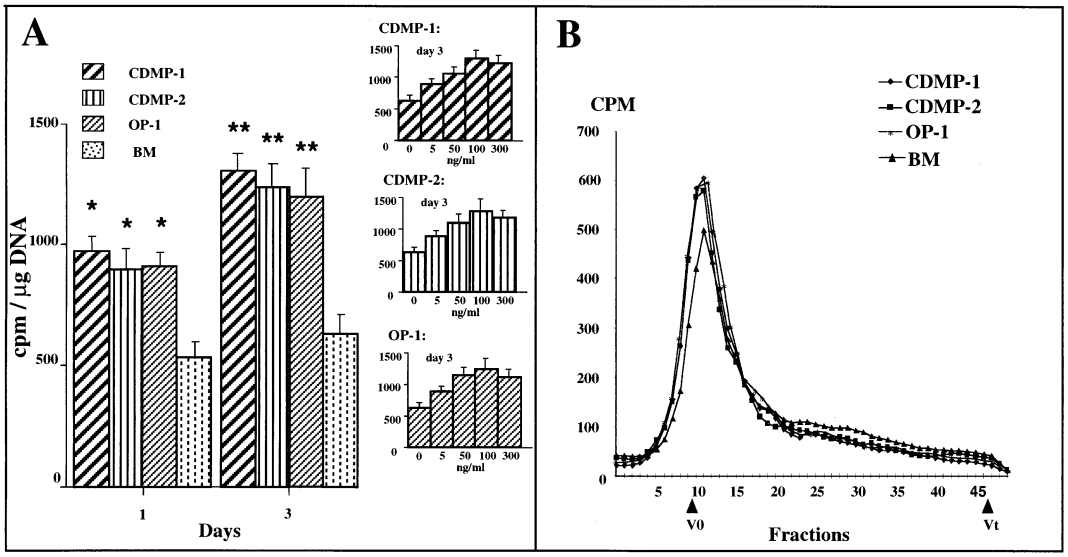

FIG. 4. [^{35}S]sulfate incorporation and analysis of the hydrodynamic size of newly synthesized macromolecules in primary fetal chondrocyte cultures. (A) Quadruplicate cultures of chondrocytes were treated for 24 and 72 h with CDMP-1 (100 ng/ml), CDMP-2 (100 ng/ml), or OP-1 (100 ng/ml). Cell cultures were labeled with [^{35}S]sulfate for 6 h, and the incorporated radiolabel was calculated per microgram of DNA content. Dose–response data were performed with four doses of CDMP-1, CDMP-2, and OP-1 and are shown in insets. The bars represent the means and standard deviations of the means. Experiments were repeated three times. (B) For the analysis of the hydrodynamic size of newly synthesized macromolecules, [^{35}S]-labeled materials were applied to a Sephacryl S-500 HR column. $^{*}p < 0.03$ versus BM. $^{**}p < 0.03$ versus BM and $p < 0.04$ versus day 1.

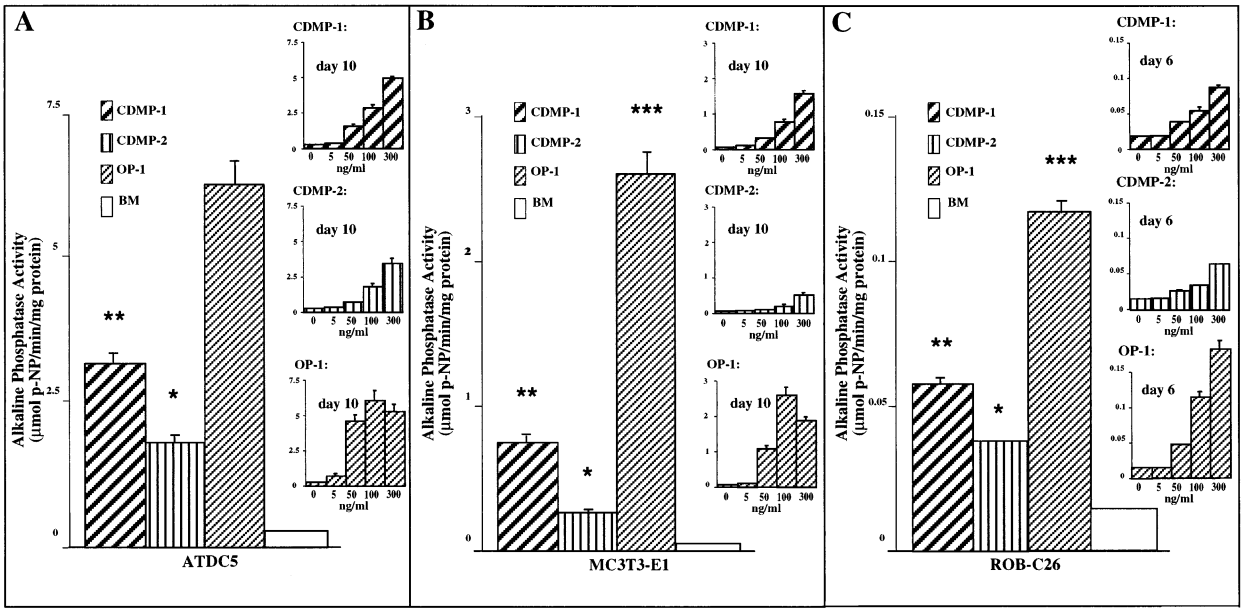

FIG. 5. Effect of CDMP-1, CDMP-2 and OP-1 on ALP activity of ATDC5, MC3T3-E1, and ROB-C26 cells. Quadruplicate cultures of (A) ATDC5, (B) MC3T3-E1, and (C) ROB-C26 cells were grown in the presence or absence of CDMP-1 (100 ng/ml), CDMP-2 (100 ng/ml), or OP-1 (100 ng/ml). Dose–response data with four doses of CDMP-1, CDMP-2, and OP-1 are shown in insets. The error bars indicate the standard deviations of the means of four observations: $***p < 0.03$ versus CDMP-1, CDMP-2, and BM; $**p < 0.03$ versus CDMP-2 and BM; and $*p < 0.03$ versus BM.

albumin (BSA) as standard (Bio-Rad, Richmond, CA, U.S.A.).

Proteoglycan biosynthesis

Rates of [^{35}S]sulfate incorporation into macromolecules were evaluated as described.[11] Briefly, cell cultures were labeled with 50 μCi/ml of [^{35}S]sulfate for 6 h at 37°C. Newly synthesized [^{35}S]sulfate-labeled macromolecules of both cell extracts (4 M guanidine-HCl in 50 mM Tris, pH 7.2), and media were determined after removal of unincorporated isotope using Sephadex G-25 (PD-10, Pharmacia Biotech, Piscataway, NJ, U.S.A.) gel chromatography. The values were normalized to DNA content. To determine the size of the newly synthesized material, 500 μl aliquots of the radiolabeled fraction were analyzed on a Sephacryl S-500 HR column (1 × 30 cm; Pharmacia Biotech), previously equilibrated with 4 M guanidine HCl, 0.5% Triton X-100 in 50 mM sodium acetate buffer, pH 6.0, at a flow rate of 0.4 ml/minute. Each fraction was measured for radioactivity. A small quantity of [^{3}H]glucosamine was added as an internal elution position marker.

RNA isolation and Northern blot analysis

Total RNA was extracted using the acidic guanidine-phenol-chloroform method.[14] For Northern blot analysis, equal amounts (5 μg) of total RNA were electrophoresed on 1.2% agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH, U.S.A.). The blots were prehybridized for 30 minutes at 68°C in hybridization buffer (Express Hyb, Clontech, Palo Alto, CA, U.S.A.), and hybridization was performed for 1 h at 68°C in the same buffer with ^{32}P-labeled cDNA probes. Probes included mouse cDNAs encoding biglycan, decorin, bone sialoprotein (BSP), osteocalcin,[15] and human ALP. A probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control cDNA probe. After hybridization, the filters were washed four times in 2× sodium chloride/sodium citrate (SSC), 0.5% SDS, and twice in 0.2× SSC, 1% SDS at room temperature for 10 minutes The blots were then exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY, U.S.A.) at –70°C for up to 24 h and quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The amounts of detected transcripts were normalized to GAPDH.

Radiolabeling of CDMP-1 and CDMP-2

CDMP-1 and CDMP- 2 were iodinated with chloramine-T.[16] Both ligands were radiolabeled to an equal specific activity.

Affinity cross-linking and immunoprecipitation

Cells were incubated 3 h on ice in binding buffer (PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 0.1% BSA) in the presence of 200–400 pM of iodinated ligand. After incubation, the cells were washed and cross-linking was performed using 1 mM bis(sulfosuccinimidyl)suberate (Pierce Chemical Co., Rockford, IL, U.S.A.) and 0.28 mM disuccinimidyl suberate for 15 minutes. The cells were

washed, scraped off the plates, centrifuged, resuspended in solubilization buffer, and incubated for 20 minutes on ice. Immunoprecipitation of the cross-linked materials was performed as described.[17] Two T 75 flasks of subconfluent cells were used for each immunoprecipitation.[18] The immune complexes were eluted by boiling for 3 minutes in SDS sample buffer containing 10 mM dithiotreitol and separated by SDS-PAGE. These gels were then dried and followed by Bio-Imaging analysis (Fuji, BAS2000; Fuji, Tokyo, Japan) or autoradiography.

Transcriptional activation response assay

Chemically mutagenized Mv1Lu cells were cotransfected with a p3TP-Lux promoter/reporter construct, BMPR-II, and plasmids containing various type I receptor cDNAs. One day after transfection, cells were treated for 20 h with CDMP-1 or CDMP-2 under serum-free conditions. Luciferase activity in the cell lysate was analyzed using the luciferase assay system (Promega Biotech, Madison, WI, U.S.A.) according to the manufacturer's protocol and a luminometer (MGM Instruments, Hamden, CT, U.S.A.).

Statistical analysis

Statistical significance was defined as a p-value < 0.05 with the Mann–Whitney U-test.

RESULTS

Production of recombinant CDMP-1 and CDMP-2

The mature domains of *cdmp-1* and *cdmp-2* were expressed in *E. coli,* refolded, and purified using reverse phase HPLC (data not shown). Aliquots (5 μg) of the purified protein preparations were analyzed by SDS-PAGE before and after reduction and alkylation (Fig. 1). The nonreduced proteins migrated as a major band around 28 kDa, while the reduced fractions showed a band at 16 kDa. These findings are consistent with appropriate homodimer formation.

CDMP-1 and CDMP-2 induce cartilage and bone formation in vivo

Histologic evaluation of the in vivo bioassay showed that implantation of the carrier without the addition of CDMPs did not initiate cartilage or bone formation (Fig. 2A). CDMP-1, CDMP-2, and OP-1 induced islands of chondrocytes 10 days after implantation (Fig. 2B). Furthermore, Von Kossa staining indicated ongoing de novo mineralization (Fig. 2C). At day 21, bone formation was apparent in the implants (Fig. 2D). A dose response experiment was performed for CDMP-1 and CDMP-2 and biochemical analysis of day 10 implants produced comparable, dose-dependent increases in ALP activity (Fig. 3). However, CDMPs were significantly less active in this in vivo assay than OP-1 ($p < 0.03$; Fig. 3, inset).

CDMP-1,CDMP-2, and OP-1 equally stimulate proteoglycan synthesis in primary chondrocytes

CDMP-1, CDMP-2, and OP-1 increased [^{35}S]sulfate incorporation into proteoglycans equally. This effect was apparent in both fetal chondocytes (Fig. 4A) and postnatal articular chondrocytes (data not shown) and was concentration- and time-dependent. Analysis of the hydrodynamic size of the newly synthesized macromolecules demonstrated the presence of the large proteoglycan species, which eluted in the void volume of a Sephacryl S-500 HR column, consistent with a cartilage phenotype (Fig. 4B). Cell proliferation, as measured by DNA content, was not affected by growth factor treatment (data not shown).

CDMPs are less osteogenic than OP-1 in osteoblast-like cell lines

We analyzed the expression of osteogenic markers in the mouse osteo/chondroprogenitor cell line ATDC5, the osteoblastic mouse clonal cell line MC3T3-E1, and the rat osteoprogenitor-like cell line ROB-C26 to determine whether CDMPs promote osteogenic differentiation. Treatment with both CDMP-1 and CDMP-2 resulted in an increase of ALP activity when compared with basal medium in all the cell lines ($p < 0.03$) (Fig. 5). This effect was significantly less with CDMP-2 than CDMP-1 ($p < 0.03$). In contrast, OP-1 was 2- to 3-fold more stimulatory than CDMP-1 ($p < 0.03$). Interestingly, in human fetal chondrocytes (52-79 days), OP-1 increased ALP values more than 10-fold, whereas CDMP-1 and CDMP-2 only slightly enhanced this activity (Fig. 6A). In the myoblast cell line C2C12, osteogenic differentiation was promoted by OP-1 but not by the CDMPs (Fig. 6B).

Further analysis of osteogenic differentiation was performed using Northern blot analysis of osteogenic markers. In ATDC5 cells, cultured in the presence of the indicated morphogens, OP-1 and CDMP-1 increased ALP mRNA levels (Fig. 7). In addition, transcripts for BSP were strongly up-regulated after OP-1 treatment, while CDMP-1 was less potent in this regard (Fig. 7). CDMP-2 only slightly enhanced BSP expression (Fig. 7). The expression of osteocalcin mRNA was not detected (data not shown). Northern blot analysis for the small proteoglycan species biglycan and decorin showed that OP-1 up-regulated decorin expression (Fig. 7). These data support differential regulation of osteogenic differentiation by OP-1, CDMP-1, and CDMP-2.

CDMP-1 and CDMP-2 bind to BMPR-IB and BMPR-II in nontransfected cells

To identify which type I and type II receptors have affinity for CDMP-1 and CDMP-2, binding studies were performed using ^{125}I-labeled CDMPs in various cell lines and in primary chondrocytes. ROB-C26 cells were affinity-labeled with iodinated CDMP-1 and CDMP-2, and the cross-linked complexes were immunoprecipitated using antisera to a panel of type I and type II receptors and analyzed by SDS-PAGE under reducing conditions. Cross-linked complexes of 80-90 kDa could be immunoprecipitated by

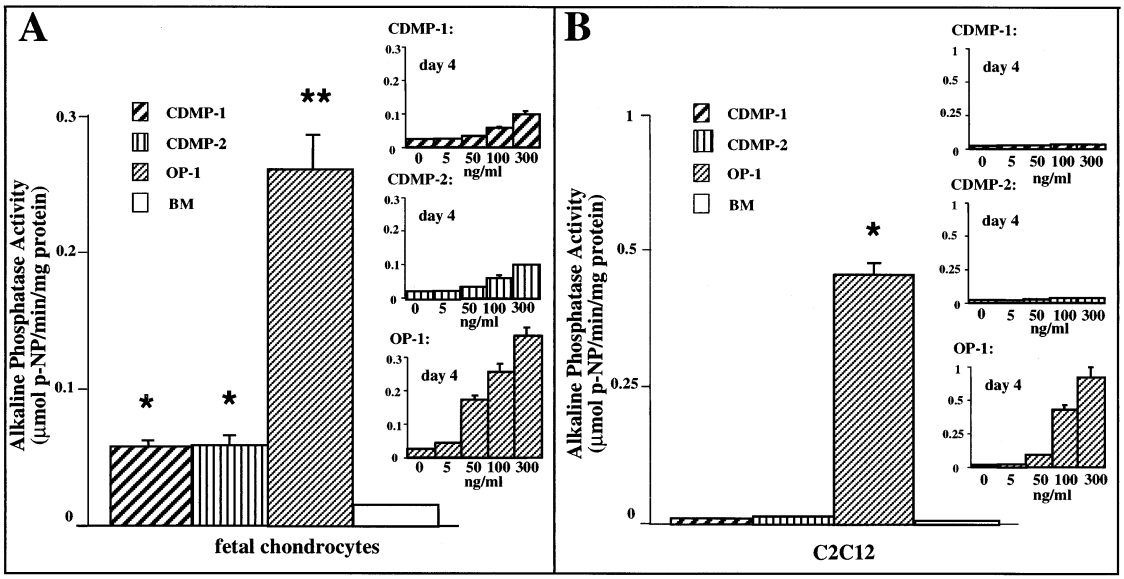

FIG. 6. ALP activity in primary fetal chondrocytes and C2C12 cells treated with CDMP-1, CDMP-2, and OP-1. Quadruplicate cultures of (A) primary fetal chondrocytes and (B) C2C12 cells were cultured in the presence or absence of CDMP-1 (100 ng/ml), CDMP-2 (100 ng/ml), or OP-1 (100 ng/ml) under serum-free conditions. The specific activity of ALP was quantitated after 4 days of treatment. Dose–response data with four doses of CDMP-1, CDMP-2, and OP-1 are depicted in insets. The bars represent the means and standard deviations of the means. Experiments were repeated three times. (A) $^{*}p < 0.03$ versus BM and $^{**}p < 0.03$ versus CDMP-1, CDMP-2, and BM. (B) $^{*}p < 0.03$ versus CDMP-1, CDMP-2, and BM.

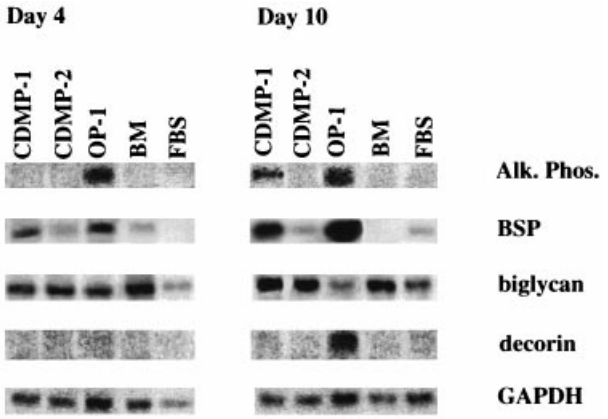

FIG. 7. Northern analysis of CDMP-1, CDMP-2, and OP-1-treated samples on the expression of osteogenic markers in ATDC5 cells. Cells were cultured with or without CDMP-1 (100 ng/ml), CDMP-2 (100 ng/ml), OP-1 (100 ng/ml), or 5% FBS for 4 and 10 days. Total RNA from each culture (5 μg) was separated on 1.2% agarose formaldehyde-agarose gels, blotted, and subsequently hybridized with the respective cDNA probes as described in Materials and Methods. GAPDH expression levels are shown to verify equal loading of mRNA (bottom).

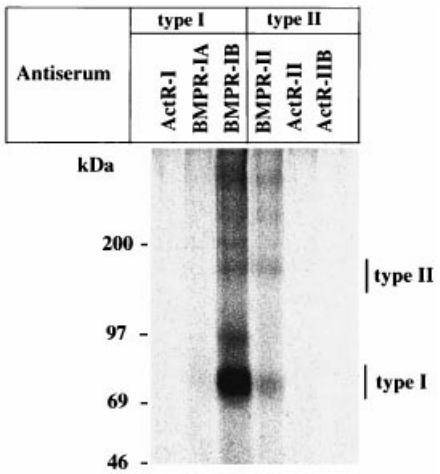

FIG. 8. Binding of CDMP-2 to type I and type II receptors in ROB-C26 cells. Binding and affinity cross-linking of ^{125}I-radiolabeled CDMP-2 were performed using ROB-C26 cells, followed by immunoprecipitation using antisera against type I and type II receptors. Samples were analyzed by SDS-PAGE and autoradiography using Bio-Imaging Analyzer.

antisera to BMPR-IB and BMPR-II for both CDMP-1 and CDMP-2. Their binding characteristics were similar, and one representative autoradiograph is shown in Fig. 8. A high molecular weight complex of 150–200 kDa, which may represent a type II receptor complex, was coimmunoprecipitated by the BMPR-IB (Fig. 8). Coimmunoprecipitation of the type I receptor complex was also detected by the BMPR-II antiserum (Fig. 8). A weak band could be seen after immunoprecipitation with the antiserum against

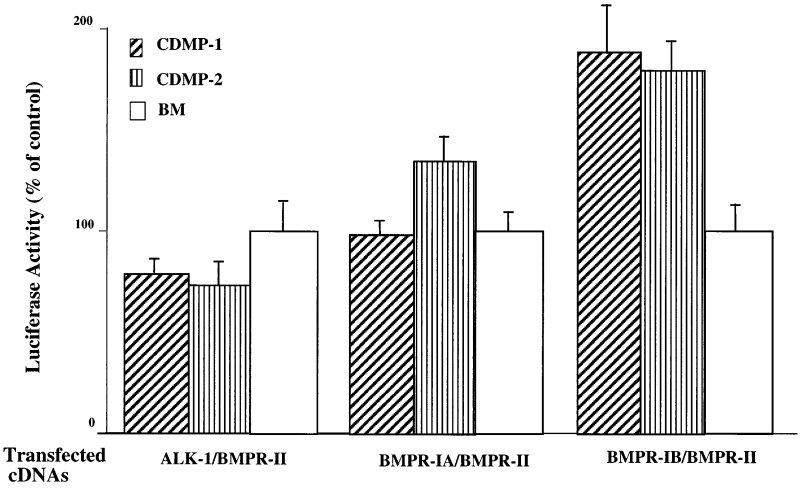

FIG. 9. Signal transduction by CDMP-1 and CDMP-2 in transfected R mutant Mv1Lu cells. (A) p3TP-Lux promoter/reporter construct was co-transfected with a cDNA for BMPR-II and plasmids containing activin receptor-like kinase (ALK)-I (negative control), BMPR-IA, or BMPR-IB into R mutant Mv1Lu cells. One day after transfection, cells were treated for 20 h with CDMP-1 (300 ng/ml) or CDMP-2 (300 ng/ml) followed by the measurement of the luciferase activity in the cell lysates. The bars represent the means and standard deviations of the means of three independent experiments.

BMPR-IA, whereas antisera against activin receptor (ActR)-I, ActR-II and ActR-IIB did not immunoprecipitate any appreciable amounts of CDMP cross-linked complexes (Fig. 8).

CDMPs transduce a transcriptional activation signal by BMPR-IB and BMPR-II in transfected cells

To investigate whether CDMP-1 and CDMP-2 are able to transduce a signal upon binding to their respective type I and type II receptor complexes, the signaling activity of CDMPs was analyzed in R mutant Mv1Lu cells using the p3TP-Lux promoter/reporter construct. The BMPR-IB/BMPR-II complex mediated an efficient signal for both CDMPs, which was in agreement with our binding data (Fig. 9). Furthermore, CDMP-2 but not CDMP-1 transduced a weak signal through the BMPR-IA/BMPR-II complex (Fig. 9). No activation was observed with other type I receptors in the complex. Cells transfected with either type I or type II receptors only did not respond to the CDMPs (data not shown).

DISCUSSION

Our study compared for the first time the activity of recombinant CDMP-1, CDMP-2, and OP-1 in the initiation and progression of chondrogenic and osteogenic differentiation both in vivo and in vitro. Our findings suggest that the CDMPs are equipotent in stimulating cartilage matrix synthesis when compared with OP-1, but have markedly reduced activity in the promotion of osteogenesis. Furthermore, CDMP-2 appeared to be less osteogenic than CDMP-1 in vitro. In addition, our data indicate that the underlying molecular basis for the differential biological responses between CDMP-1, CDMP-2, and OP-1 might be their relative affinities for specific receptor complexes.

The in vivo assay for ectopic induction of cartilage and bone formation, using rat bone residue as the delivery system, confirmed that CDMP-1 and -2 are not only structurally but also functionally related to BMPs 2, 4, 5, 6, 7.[19,20] Ectopic bone induction for Gdf-5/CDMP-1 has previously been reported.[20] Our data showed that CDMP-1 and CDMP-2 equally induce cartilage and bone formation. Interestingly, CDMPs appear to be significantly less active in this in vivo assay than OP-1. It is conceivable that the use of other carriers might affect the outcome of the cartilage and bone-inducing properties of the morphogens.

We studied the promotion of cartilage differentiation by CDMP-1 and -2 in vitro using primary cell cultures. The enhanced de novo proteoglycan aggrecan synthesis in primary chondroblasts originating from fetal limbs, as well as in postnatal articular chondrocyte cultures, supports the stimulatory role of the CDMPs in chondrogenic differentiation. It is of note that we were unable to detect differences in type II collagen expression levels by Northern analysis under the culture conditions described (data not shown). This may be due to already high expression levels at the start of the culture, because we are mainly enhancing an already existing cartilage phenotype. Only in low density monolayer cultures could we detect an up-regulation of this chondrogenic marker in the presence of CDMPs or OP-1 (data not shown). No differences in the stimulation of cartilage matrix synthesis were observed between CDMP-1, CDMP-2, and OP-1 treatments. Enhancement of matrix synthesis in chondrocyte cultures has been reported for other BMPs previously.[11,21,22] These and our data suggest that in these cell cultures BMPs/CDMPs may use the same signaling pathways regulating cartilage matrix synthesis. In contrast, using ALP activity as a marker for chondrocyte maturation/bone formation, the CDMPs are not, or only to a limited extent, affecting this marker when compared with OP-1. To explore further a difference in the promotion of the progression in the osteogenic lineage, we found that CDMPs were significantly less osteogenic than OP-1 in various osteoblast-like cell lines. It is noteworthy that the osteogenic differentiation induced by OP-1 as observed in ATDC5 cells, is associated with an increase of BSP and decorin, reflecting a full commitment in the osteogenic lineage with bone matrix deposition. The modest osteogenic activity of CDMP-1 is in contrast to previously published data, reporting no effect of Gdf-5, the mouse ho-

molog of CDMP-1, on osteoblastic cells cultured in serum-containing media.[17,20] This difference may be due to the fact that we used a chemically defined serum-free medium. Indeed, we have demonstrated that biological responses to BMPs in in vitro models are diminished in the presence of FBS.[23] In addition, this low osteogenic activity of the CDMPs was most pronounced in C2C12 cells, where no increase in ALP activity by CDMPs was observed, whereas OP-1 and as previously described BMP-2 convert these cells into the osteoblastic lineage in low serum conditions.[24] Therefore, the combined data suggest that the signaling cascade(s) with regard to osteogenesis may be distinct from these involved in cartilage differentiation, and that different members of the BMP family signal through specific pathways. In addition, our Northern analysis data in ATDC-5 cells provide further evidence that CDMP-1 and CDMP-2, although highly related (80% sequence identity in the mature biologically active region) may have distinct biological profiles depending on the cell populations.

In a first attempt to identify the molecular basis for specific biological activities elicited by various BMPs/CDMPs, in particular chondrogenesis and osteogenesis, we set out to characterize the receptor complexes for the CDMPs. We demonstrated in this manuscript that CDMP-1 and CDMP-2 bind predominantly to the BMPR-IB and BMPR-II receptors. In addition, our transcriptional response data showed that CDMPs signal through this BMPR-IB/BMPR-II complex. This is in agreement with the findings that Gdf-5 binds and signals through this complex.[17] Recently, using the in vivo developing chick limb model and in vitro micromass cultures, Kawakami et al. found that BMPR-IB and BMPR-II are the critical receptor complexes in chondrogenesis of limb mesenchymal cells.[25] These authors demonstrated intense expression of BMPR-IB in the developing limb, and showed that a dominant negative BMPR-IB receptor markedly inhibited chondrogenesis as measured by cartilage nodule formation and de novo proteoglycan synthesis. Taken together, the preferential binding of the CDMPs to the BMPR-IB/BMPR-II complex and the evidence of the direct association of this complex with chondrogenesis provides a molecular cascade for the stimulation of chondrogenic differentiation by the CDMPs. In contrast, OP-1 binds and signals through other receptor complexes besides BMPR-IB/BMPR-II.[6,7] Therefore, our data further support the possibility of the existence of distinct molecular pathways associated with osteogenesis and chondrogenesis.

A potential limitation in the interpretation of the data relates to the preparation method of the recombinant proteins because the CDMPs are bacterially refolded proteins while OP-1 was produced in mammalian cells.[19] Contaminants or the presence of inappropriately folded proteins in the CDMP preparations may affect their biological responses. However, we have consistently found the same results using at least three different batches of HPLC-purified proteins. In addition, our data show that the CDMPs and OP-1 have identical effects in dose response experiments on the proteoglycan synthesis in human fetal chondrocyte cultures, while only OP-1 stimulates ALP levels in the same cultures. Finally, the differential responses for CDMP-1 when compared with CDMP-2, most strikingly observed in ATDC-5 cells (Fig. 7), makes it unlikely that the distinct biological responses are due to different protein preparation methods.

In conclusion, we reported the expression of functional recombinant CDMP-1 and CDMP-2 and their biological activities in chondrogenic and osteogenic models in vivo and in vitro. The data show a weak osteogenic potential of the CDMPs when compared with OP-1. This differential response may be due to specific ligand–receptor interactions. Our studies suggest that the BMPs/CDMPs may provide powerful experimental tools to further unravel the mechanisms leading to cartilage or bone formation.

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